Antisense Oligonucleotide Inhibition of Bcl-xL and Bid Expression in Liver Regulates Responses in a Mouse Model of Fas-Induced Fulminant Hepatitis

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ABSTRACT

Activation of the cell-surface receptor Fas can lead to apoptosis in parenchymal cells in the liver, and if severe enough, result in fulminant hepatic failure and animal death. In the present study, we have examined the roles played by the Bcl-2 family members Bcl-xL and Bid in regulating this response. To do this, we have developed chemically modified 2′-O-(2-methoxy)ethyl antisense inhibitors of both Bid and Bcl-xL expression. In Balb/c mice, dosing with these antisense oligonucleotides reduced expression of the targeted mRNA by greater than 80% in the liver. This reduction was highly dependent upon oligonucleotide sequence and oligonucleotide dose. Reduction of Bcl-xL expression resulted in a potentiation of Fas-mediated apoptosis in liver and significant increase of the lethality of Fas-mediated fulminant hepatitis (p < 0.0001). In contrast, reduction of Bid expression protected the animals against Fas-mediated fulminant hepatitis and death (p < 0.0001). Simultaneous dosing of mice with Bcl-xL and Bid-targeting antisense oligonucleotides resulted in an inhibition of expression of both targeted proteins and protection of the animals from Fas-mediated apoptosis. These results demonstrate, for the first time, the role of Bcl-xL in regulating responses to proapoptotic Fas signaling in mouse liver. In addition, this is the first reported example demonstrating the ability of antisense inhibitors to reduce expression of multiple proteins in animals by simultaneous dosing.

Apoptosis, or programmed cell death, functions to control many biological processes, and abnormal expression or activation of the genes that regulate apoptosis is believed to play a role in maintaining the pathology of a number of diseases. Of the genes that are known to regulate apoptosis, the Bcl-2 family of proteins is perhaps the most well studied (Adams and Cory, 1998; Korsmeyer, 1999; Hengartner, 2000). In mammalian cells, members of this family that can either inhibit apoptosis (Bcl-2, Bcl-xL, Bcl-w, A1, Mcl-1, and Boo) or promote apoptosis (Bax, Bak, Bok, Bik, Bad, and Bid) have been identified (Pellegrini and Strasser, 1999). Although similarities in protein structure suggest that these genes may have overlapping and redundant functions, experimental evidence would suggest that this may not always be the case. For example, both the expression profiles and the regulation of expression of the different family members are quite discordant in cells derived from different tissues. In addition, genetic knockouts of some members of the family give rise to contrasting phenotypes. For example, Bcl-2-deficient mice are viable but demonstrate defects in hematopoiesis and homeostasis (Veis et al., 1993), whereas Bcl-xL-deficient mice die in uterus of neuronal and hematopoietic defects (Motoyama et al., 1995). These data would suggest selective and specific functions for the family members, which may be dependent upon a number of factors. These may include either subtle changes in structure between different member proteins or contextual differences due to the differential expression of other proteins involved in the apoptotic signaling process in different cellular environments. Defining specific functional roles for Bcl-2 family members in animals has proven difficult. The two most widely used approaches have been either transgenic mice, which overexpress proapoptotic proteins, or genetic knockouts. Although both approaches are very useful and provide valuable information, they both have drawbacks. For example, overexpression studies give insight into which function a gene is capable of performing when expressed (often to high levels) in a given tissue; however, they do not necessarily give information on the function of the endogenously expressed and normally functioning gene. In contrast, genetic knockouts may provide this kind of information but are sometimes embryogenically
lethal (as in the case of Bcl-xL), thus precluding studies in adult animals.

We are interested in the signaling pathways activated and the in vivo phenotype produced by the tumor necrosis factor receptor family member Fas, and whether Bcl-2 family members play critical roles in this process. Fas is highly expressed in liver on hepatocytes, and activation of Fas in vivo leads to massive hepatocyte apoptosis and can result in animal death. The roles played by individual members of the antiapoptotic members of the Bcl-2 family of proteins in regulating responses to signaling through the Fas in the liver are somewhat controversial. Overexpression of Bcl-2 in hepatocytes has been shown to protect mouse from Fas-mediated hepatic apoptosis triggered by a Fas cross-linking antibody (Lacronique et al., 1996; Rodriguez et al., 1996; Van Molle et al., 1999). However, these studies have been questioned in experiments where Fas was activated by dosing mice with a recombinant FasL preparation (Huang et al., 1999). Overexpression of Bcl-xL has also been reported to inhibit Fas-induced apoptosis in hepatocytes (de la Costa et al., 1999). We have found that Bcl-xL is abundantly expressed in mouse liver and therefore may play a normal, physiological role in regulating hepatocyte apoptosis triggered by Fas cross-linking. In contrast, the proapoptotic role of Bid in hepatocytes is fairly well defined. Bid is highly expressed in hepatocytes, and Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis (Yin et al., 1999).

To study the role of endogenously expressed Bcl-xL and the relationship of this protein with Bid in regulating liver apoptosis, we have taken advantage of the recently described chemically modified 2′-O-(2-methoxy)ethyl (2′-O-MOE) chimeric class of antisense oligonucleotides (McKay et al., 1999). After systemic dosing, antisense oligonucleotides with this modification accumulate in a number of tissues, and high levels are found in hepatocytes. When correctly designed, these antisense oligonucleotides result in a specific reduction in targeted gene expression (Zhang et al., 2000; Yu et al., 2001). Previously, we have used an antisense inhibitor with the 2′-O-MOE modification to investigate the role of Bcl-xL in cultures of human cells (Taylor et al., 1999a). Because the mRNA sequences targeted with this antisense are conserved between mouse and human Bcl-xL, we have used this antisense to specifically reduce Bcl-xL expression in mouse liver.

In addition, using a high-throughput approach, we have identified a potent and effective antisense inhibitor of mouse Bid expression. Down-regulation of Bcl-xL expression was found to result in a potentiation of Fas-mediated apoptosis in liver and an increase in the lethality of Fas-mediated fulminating hepatitis in mice, whereas reduced Bid expression was highly protective against Fas-mediated animal death. Inhibition of both genes simultaneously also resulted in protection of these animals from Fas-induced mortality, suggesting that the loss of Bcl-xL could be overcome by a reduction in Bid expression. These studies demonstrate, for the first time in animals, a direct role for endogenously expressed Bcl-xL in regulating Fas-mediated apoptotic signaling and suggest that Bid can antagonize this function.

**Materials and Methods**

**Cell Culture.** Mouse hepatoma cells (Hepa 1-6) and mouse b.END.3 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (Invitrogen, Carlsbad, CA) and penicillin/streptomycin (Invitrogen).

**Oligonucleotide Chemistry, Synthesis, and Identification.** 20-mer 2′-O-MOE chimeric antisense oligonucleotides were used in all experiments. These antisense oligonucleotides contain 2′-O-MOE/phosphorothioate residues flanking a 2′-oligodeoxynucleotide/phosphorothioate central region that supports RNase H-mediated cleavage of targeted mRNA in cells (McKay et al., 1999). The antisense targeting Bcl-xL (Isis 16009) is designed to hybridize to positions 581 to 601 on the human Bcl-xL transcript (accession number Z23115) and positions 447 to 466 on the murine Bcl-xL transcript (accession number U10101). The sequence of the antisense is 5′-CTACGCTTCCACGGACGTT-3′. Bolded and underlined residues indicate 2′-O-MOE-modified residues. The antisense targeting murine Bid (Isis 119935) is designed to hybridize to positions 9373 to 9392 on the murine Bid sequence (accession number AC006945_TRUNC). The sequence of the Bid-antisense is 5′-GACGATGTCCCTGGCCAGAAA-3′. To identify this oligonucleotide, a series of 78 antisense oligonucleotides designed to bind to different sequences in the murine Bid mRNA were evaluated for their ability to reduce Bid mRNA expression in mouse b.END.3 cells. The control oligonucleotides used in these studies were Isis 20292 (5′-CGACGCTACCTCTGGCATT-3′) and Isis 128425 (5′-CTCTGTCGACGCTTCTCCTGTT-3′). The other control oligonucleotide used was Isis 29848, designed to control for any chemical effects that antisense with this modification might have. This compound has a random mixture of modified bases at each position. The oligonucleotide sample was prepared by concurrent reaction of four amidites (A, G, C, and T) at each position of the chain. The methoxyxycarbonyl deoxyriboside amidites were used at positions 1 to 5 and 16 to 20, whereas deoxyriboside amidites were used at positions 6 to 15. The ratio of each amidite in the mixture was adjusted to ensure equivalent reactivities. After cleavage from support and deprotection with concentrated ammonium hydroxide, the sample was purified by passage over a G15 size exclusion column. Appropriate fractions were pooled and concentrated under reduced pressure. All oligonucleotides were synthesized using an Applied Biosystems 380B automated DNA synthesizer and purified as described previously (Baker et al., 1997).

**Transfection of Cells with Oligonucleotides.** Hepa 1-6 cells were grown in 100-mm tissue culture dishes until 70 to 80% confluent. b.END.3 cells were grown in 96-well plates. Cells were then transfected with oligonucleotides in the presence of 2.5 μg/mL DOTMA/DOPE (Lipofectin; Invitrogen) per 100 nM oligonucleotide and processed as described previously (Zhang et al., 2000).

**Animals and Oligonucleotide Dosing.** Eight-week-old female Balb/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in compliance with Isis Institutional Animal Care and Use Committee guidelines in an Association for Assessment and Accreditation of Laboratory Animal Care international-accredited facility. Mice were treated with the oligonucleotide (saline solution) or saline control in a final volume of 200 μl by i.p. injection.

**RNA Isolation, Northern Blotting, and RNase Protection.** Total RNA was harvested from cells and tissues using either the RNeasy method (Qiagen, Valencia, CA) or by extraction in guanidinium (Taylor et al., 1999b). Northern blotting analysis was performed as described previously (Taylor et al., 1999b). RNase protection assays to detect targeted mRNA expression were performed using RiboQuant kits (BD Biosciences Pharmingen, San Diego, CA) according to the manufacturer’s protocols. We routinely analyzed 20 μg of total RNA per lane and quantitated individual transcripts on a PhosphorImager (Amersham Biosciences Inc., Piscataway, NJ). Bcl-xL and Bid mRNA levels are compared with expression levels of a housekeeping gene glyceraldehyde-3-phosphate dehydrogenase or L32 (mitochondrial ribosomal protein) for sample loading control. In screening for Bid antisense inhibitor, Bid mRNA expression was also determined by TaqMan polymerase chain reaction using standard methods.
protocols (forward primer, 5′-TGTGAGGAACTTGGTTAGAAACG-3′; reverse primer, 5′-CAGCTGAACGCCGAG-3′; probe, 5′-CTCAGGACCGCCGACCGGTC-3′).

**Western Blotting.** Bcl-xL and Bid protein expression was determined by Western blotting. The antibodies used were a mouse monoclonal from BD Biosciences Pharmingen for Bcl-xL and a goat polyclonal from R & D Systems (Minneapolis, MN) for Bid. For the cleaved form of caspase 3 and caspase 7, two rabbit polyclonal antibodies (Cell Signaling Technology Inc., Beverly, MA) were used respectively. The rabbit polyclonal anti-caspase 3 antibody recognizes both 19- and 17-kDa cleaved products of caspase 3.

**Fas-Mediated Fulminant Hepatitis.** An anti-mouse Fas monoclonal antibody, Jo-2 (BD Biosciences Pharmingen), can cross-link Fas receptor and induce apoptosis in liver and animal fulminant death (Zhang et al., 2000). To examine the effects of antisense oligonucleotide treatment on Fas signaling in liver, we injected mice i.p. with Jo-2 antibody at the indicated dose level in 200 μl of phosphate-buffered saline 24 h post-treatment with antisense oligonucleotides. Animals were sacrificed at the indicated time after challenge or when they were judged to have reached a moribund state according to Association for Assessment and Accreditation of Laboratory Animal Care guidelines.

**Histology.** Liver tissue samples were fixed in 10% buffered formalin and embedded in paraffin. After preparation for immunohistochemistry section slides were stained with a rabbit polyclonal antibody recognizing cleaved caspase-3 (Cell Signaling Technology Inc.). A horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (The Jackson Laboratory) was used and followed by treatment of chromagen (3,3′-diaminobenzidine, Sigma-Aldrich, St. Louis, MO). The slides were counterstained with hematoxylin. Additional liver samples were stained with hematoxylin and eosin.

**Statistical Analysis.** F test was conducted to check the homogeneity of variance for the alanine aminotransferase data, and one-tailed two-sample t test was used to calculate p values. The product-limit or Kaplan-Meier estimator was used to analyze animal survival data. A subject was censored on the last day of follow-up, if the subject had not yet died. Survival curves from different groups were then compared using the log-rank test for computation of p values.

## Results

**Identification of Bcl-xL and Bid Antisense Oligonucleotides.** The identification and characterization of an antisense inhibitor of expression of human Bcl-xL (Isis 16009) has been described previously (Taylor et al., 1999a). The mRNA sequence targeted by this antisense oligonucleotide on the human Bcl-xL transcript is also conserved in the murine Bcl-xL transcript. To confirm that this antisense inhibits expression of murine Bcl-xL, we initially determined whether the compound could reduce Bcl-xL expression in murine hepatocyte (Hepa 1-6) cell culture. Hepa 1-6 cells were found to express high levels of both Bcl-xL mRNA and protein (Fig. 1). Inhibition of Bcl-xL and Bid Expression in Mouse Liver. To reduce expression of Bcl-xL, Isis 16009 targeting Bcl-xL, or Isis 20292 (control oligonucleotide) at a final concentration of 400 nM as described under Materials and Methods. Bcl-xL mRNA expression was determined by RNase protection assay (left) and Bcl-xL protein expression was determined by Western blotting (right).

**Identification of Bcl-xL and Bid Antisense Oligonucleotides.** The identification and characterization of an antisense inhibitor of expression of human Bcl-xL (Isis 16009) has been described previously (Taylor et al., 1999a). The mRNA sequence targeted by this antisense oligonucleotide on the human Bcl-xL transcript is also conserved in the murine Bcl-xL transcript. To confirm that this antisense inhibits expression of murine Bcl-xL, we initially determined whether the compound could reduce Bcl-xL expression in murine hepatocyte (Hepa 1-6) cell culture. Hepa 1-6 cells were found to express high levels of both Bcl-xL mRNA and protein (Fig. 1). Treatment with Isis 16009 reduced the expression of both Bcl-xL mRNA and protein by greater than 80%, confirming that the antisense is able to inhibit murine, as well as human Bcl-xL expression. In addition to Bcl-xL, Hepa 1-6 cells expressed Bcl-w, Bak, Bax, and Bad, but no detectable Bcl-2 (Fig. 1). The expression of these mRNA transcripts was not effected by treatment of cells with Isis 16009, demonstrating the selectivity of the antisense inhibitor for Bcl-xL.

To identify an active antisense inhibitor of murine Bid, we evaluated a total of 78 different antisense oligonucleotides designed to target sequences throughout the murine Bid transcript. These antisense oligonucleotides were transfected into mouse b.END.3 cells, and the levels of Bid mRNA expression were determined by TaqMan polymerase chain reaction. The oligonucleotide sequence that gave the greatest reduction in Bid expression was Isis 119935, which reduced Bid mRNA expression by greater than 80% in b.END.3 cells at 150 nM (data not shown).

**Inhibition of Bcl-xL and Bid Expression in Mouse Liver.** To reduce expression of Bcl-xL, Isis 16009 was administered to mice daily for 4 days at 12.5, 25, 50, or 100 mg/kg, and Bcl-xL mRNA expression in liver was determined by Northern blotting 24 h after the final dose. At the highest antisense dose administered, Bcl-xL mRNA expression was reduced by greater than 90% compared with saline-treated animals. A control oligonucleotide at this dose was without effect on Bcl-xL expression (Fig. 2A). The ability of Isis 16009 to specifically reduce Bcl-xL expression after activation of Fas signaling was determined by RNase protection and Western blotting. Mice were dosed with four daily doses of saline, Isis 16009 at 50 mg/kg, or a control oligonucleotide at 50 mg/kg. The Fas cross-linking Jo-2 antibody was administered 24 h after the final dose, and animals were sacrificed 4 h later. RNase protection demonstrated that in addition to Bcl-xL, mouse liver expressed Bcl-w, low levels of A-1, Bak, Bax, and Bad mRNA (Fig. 2B). No Bcl-2 transcript was detectable. Fas cross-linking was without effect on expression of these transcripts. Isis 16009 reduced Bcl-xL mRNA
expression by greater than 90% but was without effect on the expression of the other apoptotic genes measured. Western blotting of liver protein samples from the same experiment demonstrated abundant Bcl-xL protein expression (Fig. 2C), which was also unaffected by Fas cross-linking. Expression of the Bcl-xL protein was reduced by greater than 80% by Isis 16009 dosing, but was not altered by treatment with the control oligonucleotide.

A similar dosing schedule was used to demonstrate an antisense-mediated down-regulation of Bid expression in mouse liver. In these studies, Isis 119935 targeting Bid or a control oligonucleotide was administered daily for 4 days at 50 mg/kg, and both Bid mRNA expression and protein expression determined 24 h later. RNase protection demonstrated an antisense-specific reduction in Bid mRNA expression by greater than 80% with no effect on the expression of a number of unrelated genes (including PARP and Daxx) (Fig. 3). Expression of Bid protein was also specifically reduced more than 95% by treatment of the mice with the Bid antisense (Fig. 3).

We next determined whether simultaneous dosing of mice with both the Bcl-xL and the Bid antisense could inhibit expression of both of these genes. Mice were administered with saline or 50 mg/kg Isis 16009 targeting Bcl-xL. The animals receiving Isis 16009 also received either saline; Isis 119935 targeting Bid at 12, 24, or 50 mg/kg; or a control oligonucleotide at 50 mg/kg in the same injection. The oligonucleotides were administered i.p. daily for 4 days, and the animals were sacrificed 24 h after the last dose and both Bid and Bcl-xL protein expression in liver determined by Western blotting. The same protein sample was also probed for expression of β-actin as a loading control (Fig. 4). A Bid oligonucleotide-specific, dose-dependent reduction in Bid protein expression was seen, whereas a reduction in Bcl-xL expression was observed in the liver of the mice receiving the constant dose of Bcl-xL antisense. The control oligonucleotide at high dose was without effect on expression of Bid protein in the presence of Bcl-xL antisense.

**Effects of Either Bcl-xL or Bid Reduction on Fas-Mediated Apoptosis and Fulminant Hepatitis.** To evaluate the impact of antisense-mediated reduction of Bcl-xL

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**Fig. 2.** Effect of antisense oligonucleotides on Bcl-xL expression in mouse liver. A, mice were dosed with saline, Isis 16009 targeting Bcl-xL, or Isis 20292 (control oligonucleotide) (daily × 4, i.p. at the indicated dose, in milligrams per kilogram). Twenty-four hours after the last dose animals were sacrificed and total RNA extracted from liver. The expression of Bcl-xL mRNA was determined by Northern blotting. Each lane represented the sample from individual animal. The gel was stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase expression to confirm equal RNA loading. B, mice were dosed with saline, Isis 16009 targeting Bcl-xL, or Isis 20292 (control oligonucleotide) (daily × 4, i.p. at 50 mg/kg). Twenty-four hours after the last dose, animals received either saline or 3 μg of Jo-2 antibody (i.p.). Four hours later animals were sacrificed and total RNA extracted from liver. Bcl-xL (and other apoptotic gene) expression was determined by RNase protection assay. C, mice were dosed as in B and Bcl-xL protein expression in liver determined by Western blotting as described under Materials and Methods.

**Fig. 3.** Effect of antisense oligonucleotides on Bid expression in mouse liver. Mice were dosed with saline, Isis 119935 targeting Bid, or Isis 128425 (control oligonucleotide) (daily × 4, i.p. at 50 mg/kg). Twenty-four hours after the last dose, animals were sacrificed and total RNA extracted from liver. The expression of Bid mRNA was determined by RNase protection assay (left). Bid protein expression was determined by Western blotting (right). Each lane represented sample from individual animal.
expression in mouse liver on Fas-mediated fulminant hepatitis, three Jo-2 antibody doses were used to induce liver damage. In the first experiment, 7 μg of the Fas-activating Jo-2 antibody was used. Previous studies (data not shown) had determined that this dose of antibody represents an LD₉₀ dose in mice. When mice were pretreated with either saline, or the control oligonucleotide (50 mg/kg × four daily doses), with a final dose 24 h before administration of Jo-2 antibody we found that 90 to 100% of the animals died within 12 h (Fig. 5A). In contrast, animals treated with the Bcl-xL antisense oligonucleotide before the Jo-2 administration were all dead within 4 h of antibody dosing (Fig. 5A). The death in Bcl-xL antisense-treated group was significantly earlier than that in saline-treated group (p < 0.0001), whereas the timing of death between saline- and control oligonucleotide-treated groups was indistinguishable (p = 0.9248). This sensitizing effect on lethality was also dependent on the Bcl-xL antisense doses (Fig. 5B).

In a second series of studies, mice were treated with a dose of 4.5 μg of Jo-2 antibody representing an approximate LD₁₀. Pretreatment of the mice with either saline or a control oligonucleotide (50 mg/kg × four daily doses) with the final dose 24 h before antibody administration were not significantly different (p = 0.1464) in the numbers of animals that died from Jo-2 administration (Fig. 5C). In contrast, animals that received the Bcl-xL antisense suffered 80 to 90% mortality within 12 h (Fig. 5C) (p = 0.0006 compared to the saline group). Four hours after receiving a dose of 4.5 μg of the Jo-2 antibody, the mouse livers exhibited a morphology typical of injury due to hepatocyte apoptosis as has been described elsewhere (Fig. 6B) (Ogasawara et al., 1993). Numerous small hemorrhaged foci were apparent, and many hepatocytes had condensed nuclei. A similar pattern was seen in mice that received the control oligonucleotide before dosing with the Jo-2 antibody (Fig. 6D). In contrast, animals that had received the Bcl-xL antisense before dosing with the Jo-2 antibody demonstrated much more severe liver damage (Fig. 6C). The extent of hemorrhaged damage was extensive, with numerous red blood cells apparent. Few intact hepatocytes not undergoing apoptosis could be found. The effects of a sublethal dose of Jo-2 antibody (3 μg) were also evaluated on serum alanine aminotransferase (ALT). In sera of the mice treated with saline, Isis 16009 targeting Bcl-xL, or a control oligonucleotide as described above, ALT was significantly increased in all groups injected with 3 μg of Jo-2 antibody at 12 h after challenge compared with saline. However, the Bcl-xL antisense treatment resulted in a more prom-
Fig. 6. Effect of Bcl-xL targeting antisense oligonucleotide on Fas antibody mediated-liver damage. Mice were dosed with saline (A and B), a Bcl-xL-targeting antisense oligonucleotide (Isis 16009) (C), or a control oligonucleotide (Isis 20292) (D) (daily × 4, i.p. at 50 mg/kg). Twenty-four hours after the last dose, animals received 4.5 μg of Jo-2 antibody (B–D) or saline (A). Four hours later, animals were sacrificed and livers processed for H&E stain as described under Materials and Methods. E, mice were treated as above and challenged with 3 μg of Jo-2 antibody (i.p.). Twelve hours later, sera were collected, and ALT activity was determined (Biomedical Testing Services, San Diego, CA.) Error bars represent standard error, n = 4.
Caspases 3 and 7 are downstream caspases that are activated and cleaved subsequent to Fas activation. To determine the kinetics of this response in liver, mice were treated with 3 μg of Jo-2 antibody and sacrificed at different times subsequently. Caspase activation was determined by Western blotting using antibodies that recognize the cleaved forms of either caspase 3 or caspase 7. The cleaved forms of the two caspases were apparent as early as 4 h after dosing with 3 μg of the Jo-2 antibody and were much more prominent at later time point, 6 h (Fig. 7A). We next treated mice with the Bcl-xL antisense to reduce Bcl-xL expression and then dosed with 3 μg of Jo-2 antibody and examined caspase 3 and 7 cleavage by Western blotting. Four hours after Jo-2 antibody injection, the levels of the cleaved forms of both caspases were much greater in livers of the animals treated with Bcl-xL antisense than treated with saline or control oligonucleotide (Fig. 7B). Immunohistological staining for cleaved caspases 3 and 7 indicated similar activation in liver (data not shown).

In contrast to the results obtained with the Bcl-xL antisense, mice that were dosed with the Bid antisense (25 mg/kg × 4 daily doses, final dose 24 h before administration of 7 μg of Jo-2 antibody) were all alive 48 h after administration of the Jo-2 antibody (Fig. 8) (p < 0.0001 compared with saline group). These data are consistent with results obtained using Bid-deficient mice that are quite resistant to Fas-induced hepatocellular apoptosis and animal death (Yin et al., 1999).

Finally, we explored the relationship between Bcl-xL and Bid in regulating Fas-induced mortality by dosing with Bcl-xL and Bid antisense oligonucleotides in combination. When mice were dosed with both Bcl-xL and Bid antisense oligonucleotides at 50 mg/kg each (four daily doses), no animals were dead 24 h after administration of 7 μg of Jo-2 antibody (Fig. 9A) (p < 0.0001 compared with saline group; the death of control oligonucleotide-treated group was indistinguishable from saline-treated, p = 0.2100). This indicated that the down-regulation of Bid could overcome the loss of Bcl-xL. The protective effect of Bid down-regulation is highly dependent upon antisense dose. Mice were dosed with either saline or the Bcl-xL targeting antisense (50 mg/kg × 4 daily doses) combined with either the Bid targeting antisense (at either 6, 20, or 50 mg/kg × 4 daily doses) or a control oligonucleotide (50 mg/kg × 4 daily doses). Mice were then administered 7 μg of Jo-2 antibody and mortality monitored. As demonstrated previously, 9 of 10 animals that were pre-treated with saline died within 11 h (Fig. 9B). Bcl-xL antisense treatment alone, or in combination with a control antisense, potentiated the effects of Jo-2 treatment and resulted in more rapid mortality, whereas mice that received the Bid antisense in combination with the Bcl-xL antisense exhibited extended survival times dependent on the dose of Bid antisense oligonucleotide (p < 0.0001; extended when 6 mg/kg of Bid antisense added; p = 0.0007, further extended when 20 mg/kg of Bid antisense added). Animals receiving the higher doses of Bid antisense (20 and 50 mg/kg) survived through 2 days and 8 days, respectively.

The antagonistic effect of Bid on Bcl-xL was also examined histologically. Mice were treated at four daily doses with saline, Bcl-xL targeting antisense at 50 mg/kg or Bid targeting antisense alone at 12 mg/kg, or a combination of both at 50 and 12 mg/kg, respectively, or a control oligonucleotide at 50 mg/kg. Livers were then taken from mice sacrificed 3.5 h after injection with 7 μg of Jo-2 antibody. Consistent with the
Bcl-xL and 12 mg/kg of Bid antisense together, and 12 mg/kg Bid antisense alone showed that Bid antisense treatment antagonized the effect of Bcl-xL inhibition on the extent of caspase 3 cleavage.

Discussion

Fas, or CD95, has been shown to be a critical regulator of apoptosis in hepatocytes. Under normal conditions, Fas may play a physiological role in maintaining liver homeostasis, because mice deficient in Fas develop liver hyperplasia. However, aberrant Fas-mediated apoptosis in hepatocytes is believed to regulate the progression of a variety of diseases (Galle and Krammer, 1998; Benedetti and Marucci, 1999). Therefore, the effective regulation of Fas-mediated signaling and apoptosis seems to be of critical importance in maintaining normal hepatocyte function. It has been one of our goals to better understand how apoptosis in the liver is controlled, in the hope that this will lead to the development of improved therapeutics to treat diseases of the liver involving inappropriate apoptosis.

Key regulators of apoptosis are members of the Bcl-2 gene family (Adams and Cory, 1998; Korsmeyer, 1999). At least six members of the family are known to protect cells from apoptosis induced by many stimuli, and an additional 10 family members are proapoptotic (for example, Bid). Members of the family can physically interact with each other, either as hetero- or homodimers, and these interactions are believed to control apoptotic signaling to some extent. Two signaling pathways have been proposed for Fas, type I and type II (Schmitz et al., 2000). In the former, the mitochondrial Bcl-2 proteins do not seem to be necessary; in the latter, where signaling is amplified in the mitochondria, these proteins are believed to play a role.

Hepatocytes are believed to be type II cells (Peter and Krammer, 1998), and strong evidence exists for a Bid-dependent pathway, because Bid-deficient mice are somewhat resistant to Fas-induced hepatocyte apoptosis. However, the role of individual Bcl-2 family members in mediating Fas-induced cell-death in these cells is still somewhat controversial and little is known of the normal physiological regulators of apoptosis in the whole liver. To begin to address these issues, we have used RNase protection assay to identify which antiapoptotic Bcl-2 family members are expressed in mouse liver. We found high levels of expression of Bcl-xL, moderate levels of Bcl-w, and low but detectable levels of A-1 (after Fas activation). We were unable to detect any Bcl-2 transcript, and therefore we have focused on the role played by Bcl-xL in regulating apoptosis in hepatocytes. Unlike the viable Bcl-2 genetic knockout mice, Bcl-xL knockout mice die during embryogenesis (Motoyama et al., 1995), and so cannot readily be used to study Bcl-xL function in adult animals.

previous survival results, inhibition of Bcl-xL caused a significant increase in both liver hemorrhage and caspase 3 activation in the comparison with saline-treated animals, whereas inhibition of Bid was protective in both (Fig. 10). Adding Bid antisense to Bcl-xL antisense treatment resulted in a diminishment of the potentiation due to the suppression of Bcl-xL.

To further characterize this response, caspase 3 activation in liver was determined by Western analysis (Fig. 11). At 3.5 h after challenge with 7 μg of Jo-2 antibody, greater caspase 3 cleavage was apparent in the liver of mice dosed with 50 mg/kg of Bcl-xL antisense compared with those dosed with saline (4× daily dose). The treatment with 50 mg/kg Bcl-xL and 12 mg/kg of Bid antisense together, and 12 mg/kg Bid antisense alone showed that Bid antisense treatment antagonized the effect of Bcl-xL inhibition on the extent of caspase 3 cleavage.
same chemical modifications that targets Fas mRNA (Zhang et al., 2000). These results demonstrate a consistent pharmacology for chimeric antisense oligonucleotides with the 2’-O-MOE modification and suggest that this class of compound can be readily used to down-regulate hepatocyte gene expression in whole animals.

Treatment with Isis 16009 targeting Bcl-xL alone was without any obvious effects on liver morphology or apoptosis, suggesting that Bcl-xL does not play a significant role in regulating apoptosis in an unstressed liver. In fact, mice did not exhibit any obvious signs of toxicity after antisense dosing. Treatment with Isis 16009 targeting Bcl-xL alone was without any obvious effects on liver morphology or apoptosis, suggesting that Bcl-xL does not play a significant role in regulating apoptosis in an unstressed liver. In fact, mice did not exhibit any obvious signs of toxicity after antisense dosing. This is in contrast to the Bcl-xL-deficient mice that die around embryonic day 13. These animals exhibit extensive apoptotic cell death in neurons of the developing brain and spinal cord (Motoyama et al., 1995). Previous studies have shown that antisense oligonucleotides do not readily cross the blood-brain barrier, and so do not suppress target gene expression in brain (Phillips et al., 1997). Therefore, this may prevent some of the toxicity apparent in the genetic knockout mice. Alternatively, Bcl-xL may be required for embryonic development, but not required for viability of adult mice.

The effects of Fas activation in the induction of apoptosis in hepatocytes with either a 3.0- or 4.5-μg dose of Jo-2 antibody were greatly enhanced by the lack of Bcl-xL. This was apparent both as an increase in overt liver damage (increased hemorrhage foci and ALT) and increased caspase 3 and 7 cleavage. After a lethal dose of Jo-2 (4.5 or 7 μg), animals also died more quickly than those pretreated with either saline or the control antisense. The increased cleavage of caspase 3 seen during enhanced apoptosis is consistent with the proposed role for caspase 3 in mediating Fas-induced apoptosis in vivo, because caspase 3 is involved in the activation of Fas in hepatocytes (Woo et al., 1999). In fact, a model has been proposed for the existence of a positive feedback loop for Fas-induced hepatocyte apoptosis (Woo et al., 1999) that requires caspase 3-mediated cleavage of Bcl-xL and Bcl-2, which in turn suppresses their antiapoptotic function. The data described in our work identifies Bcl-xL as a central component of this pathway in hepatocytes.

Our results with mice treated with a Bid antisense oligonucleotide are entirely consistent with those previously described using Bid-deficient mice (Yin et al., 1999). Antisense-
mediated reduction in Bid expression protected liver against Fas-mediated apoptosis. We have extended these findings by targeting the expression of both Bid and Bcl-xL simultaneously. As far as we are aware, this is the first example of multiple gene knockdowns in animals using antisense, and these studies clearly demonstrate the utility of this approach for dissecting complex functional relationships between genes in regulating responses in vivo. In addition, by titrating the dose of Bid antisense given, we have been able to demonstrate for the first time a “gene-dosing” relationship between Bid and Bcl-xL. As the level of Bid protein is reduced, so is the ability of Fas cross-linking to cause fulminating hepatic failure, even when Fas apoptotic signaling is poten
tiated at suppressed levels of Bcl-xL expression.

In conclusion, we describe here the use of a class of potent, durable, and specific antisense oligonucleotides to suppress Bcl-xL and Bid expression in mouse liver. These results demonstrate for the first time 1) a role for endogenously expressed Bcl-xL, and 2) the oppositional relation between Bcl-xL and Bid expression in mouse liver. These results demonstrate for the first time 1) a role for endogenously expressed Bid and Bcl-xL, and 2) the oppositional relation between Bcl-xL and Bid functions in regulating Fas-mediated apoptosis. We have extended these findings by titrating the dose of Bid antisense given, we have been able to demonstrate for the first time a “gene-dosing” relationship between Bid and Bcl-xL. As the level of Bid protein is reduced, so is the ability of Fas cross-linking to cause fulminating hepatic failure, even when Fas apoptotic signaling is poten
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References

McKay R, Miraglia L, Cummins L, Owens S, Sasmor H, and Dean NM (1999) Characterization of a potent and specific class of antisense oligonucleotide inhibi
godeoxynucleotide (CGP 69846A) after intravenous and subcutaneous administra
Schmitz I, Kirchhoff S, and Krammer PH (2000) Regulation of death receptor-
Taylor JK, Zhang QQ, Monia BP, Marcusson EG, and Dean NM (1999a) Inhibition of Bcl-xL expression sensitizes normal human keratinocytes and epithelial cells to apoptotic stimuli. Oncogene 18:4495–4504.
Taylor JK, Zhang QQ, Wyatt JL, and Dean NM (1999b) Induction of endogenous Bcl-xL through the control of Bcl-x pre-mRNA splicing by antisense oligonu

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